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Tohoku Journal of Agricultural Research
Vol. 20, No. 3, Oct. 1969
Printed in Japan

Studies on the Infection of Cucumber Mosaic Virus VI. Process of Formation of Local Lesion

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(Received, August 6, 1969)

Summary

With the combinations of cowpea and CMV, the process of local lesion formation was observed.

On the infected portions, the physiological abnormality of the palisade cells precedes to that of the epidermal cells. Namely, the group of the shrivelled cells appears in the palisade tissue at 9–10 hours after the inoculation, and the peroxidase of the epidermal cells upon them is activated. These shrivelled cells within the palisade tissue correspond to more than 90 percent of the final necrotic cells. The group of shrivelled cells became brown within 2–3 hours after then, and the peroxidase activated cells also became brown and died. The appearance of virus activity is not recognized completely until 9–10 hours after inoculation. As mentioned above, the necrotic cells of the local lesion portion and the cells in which the virus is replicated are not formed gradually but appeared suddenly. When the inoculated copwea was treated at 34°C or immersed in water, the formation of the local lesion was extremely inhibited. As to the relation between the treated time after inoculation and infection, when these treatments were performed for less than 5 hours, infection was not effected. But infection was inhibited exceedingly with the treatment performed for over 7 hours. On the other hand, when above treatment was done after 7 hours after inoculation, infection was not effected. From the above mentioned results, the cause of infection inhibition for such a treatment is the result that the behavior of virus at 5–7 hours after inoculation was inhibited. This period seems to be the time when the secondary and tertiary infected cells are composed. Therefore, the secondary and tertiary infected cells are formed abruptly after passing a certain latent period in the primary infected cell, thus the virus replicates and the main portion of the necrosis is formed.

With the combination of a virus and a host, i.e., of cucumber mosaic virus (CMV) and cowpea, the necrotic local lesion is formed on the inoculated leaf. It has been assumed that the local lesion formation would be the result of the resistance of the host tissue dependent on a hypersensitive reaction for the invasion of the virus (1). Namely, it has been supposed that the local lesion is formed in the portion where the virus has invaded and replicated, and that the replicating virus

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is enclosed in this portion. When the leaf of *Nicotiana glutinosa* is inoculated with tobacco mosaic virus (TMV), the local lesion is formed on the inoculated leaf and TMV perticles are observed in the necrotic tissue and its vicinity, but TMV particles are not observed in the leaf tissue without the lesion (1). On the other hand, when the broad bean leaf was inoculated with alfalfa mosaic virus (AMV), the virus activity was recognized in the portion without the local lesion as well as in the local lesion.* Rappaport and Wildman reported that the size of the local lesion formed on *N. glutinosa* leaf differed by strains of TMV (2). Therefore, the mechanism of local lesion formation and its significance would differ in each case according to the combination of virus and host.

In the previous report (3), we described morphological changes of the tissue in the local lesion formed on the cowpea leaf by the infection of CMV. In this paper, we will report on the process of local lesion formation investigated successively after the inoculation and inaddition will discuss on the spreading form of virus from cell to cell.

Materials and Methods

Virus and Plant

The virus used is the ordinary strain of cucumber mosaic virus (CMV-O). The virus was allowed to replicate on tobacco leaf (Bright yellow) and purified by the method used of Scott (4). The plant used is cowpea (*Vigna sinensis* Endle var. Kurodane Sanjaku) which forms a necrotic local lesion upon infection with CMV. Cowpea and tobacco plants were grown in a Growth Cabinet (3). The primary leaves of cowpea were used on the seventh to the ninth days after sowing. After the inoculation, the inoculated cowpea leaves were cut and floated on Knop's solution in a covered Petri dish, and incubated under continuous fluorescent light of 3000 lux at 28°C.

Inoculation

The inoculum used is the purified virus which dissolved in 1/10 M phosphate buffer (pH 7.0). Inoculation was done by the carborundum method (3).

Microscopic Obserbation

The tissue was stained when the microscopic observation is performed. The fresh tissue was stained with 0.005% neutral red. The tissue was fixed by F.A.A. (formalin 2, acetic acid 6, 50% ethyl alcohol 92) for 10 minutes, was washed with water and then stained with pyronine methyl green. The peroxidase activity of the cell was observed by benzidine reaction (7).

Other methods will be described in each experiment.

* Unpublished data of our laboratory

Results

a) Morphological changes and peroxidase activity of cells in the local lesion.

The numbers of cowpea leaf cells which composed one local lesion were 30 epidermal cells, 120 mesophyll cells and 240 spongy cells in average, and the total number of these constitutive cells was about 500 as described in the previous

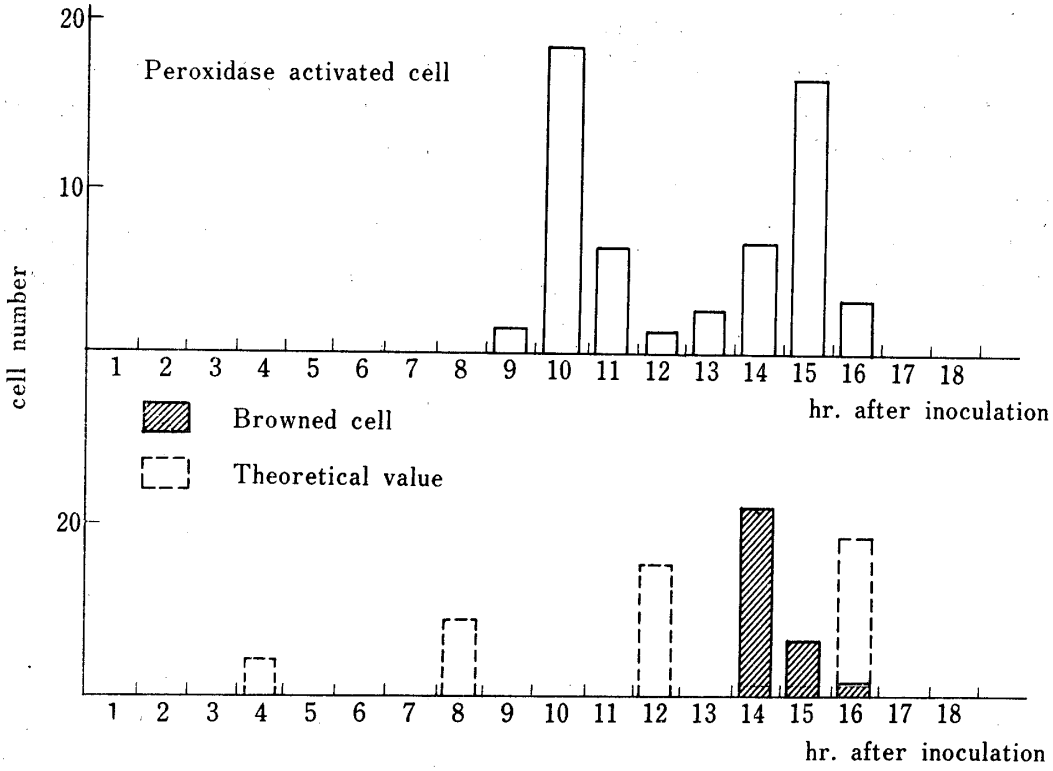


Fig. 1. Appearence of peroxidase activated cells and browned cell of epidermis in one local lesion.

Theoretical value: The value indicates an increase in the number of necrotic cells per hour after inoculation, if the virus spreads radially from one cell to surrounding cells with uniform velocity. Namely, since a local lesion could be regarded as a circle horizontally, and when the total cell number composing lesion is N, then $\sqrt{N/\pi}$ cells could form an imaginary radius (here-to-fore, termed as radial cells) from the primary infected cell as the original point. The term $\sqrt{N/\pi}$ expresses the relation of the area of a cricle ($\pi r^2=N$). When the finished local lesion is formed at T hours after inoculation and when the virus spreads radially from the primary infected cell to surrounding cells at uniform velocity, then the number of radial cell invased per one hour by the virus is $\sqrt{N/\pi}\,/T$. Therefore, the total cell number invased by the virus t hours after inoculation would be $[(\sqrt{N/\pi}\,/T)\,t]^2\pi$. But virus spread surrounding cells during a given hours (Lt), after the latent period, therefore above value is exactly $[(\sqrt{N/\pi}\,/T-Lt)\,(t-Lt)\,]^2\pi=N(t-Lt)^2/(T-Lt)^2$; $(T\geqq Lt)$ N; Final number of necrotic cell in a local lesion portion. T; Time which local necrotic lesion appeared completely. Lt; Latent period, and 28°C on the combination of CMV and cowpea it takes about 2,5 hr. (5). π; 3.14

report (3). This experiment was performed to ascertain the behavior of the virus in the infected tissue by observing periodically the morphological and physiological changes of the infected cells until the local lesion is formed after the inoculation. The inoculation was done on the lower epidermis. The inoculated lower epidermis was stripped every one hour after the inoculation and stained by pyronine methyl green and neutral red for identifying the living or dead cells, and the peroxidase activated cells were measured.

Within 8 hours after the inoculation, neither cells showing a difference by the staining, nor cells stained in blue color by benzidine reaction were observed. But at 9–10 hours after inoculation, the shrivelled cell groups occurred in the palisade tissue and the peroxidase of the epidermal cells on these mesophyll cells was activated. Numbers of these epidermal cells on which peroxidase was activated, corresponded to about 80% of the final total numbers of the necrotic epidermal

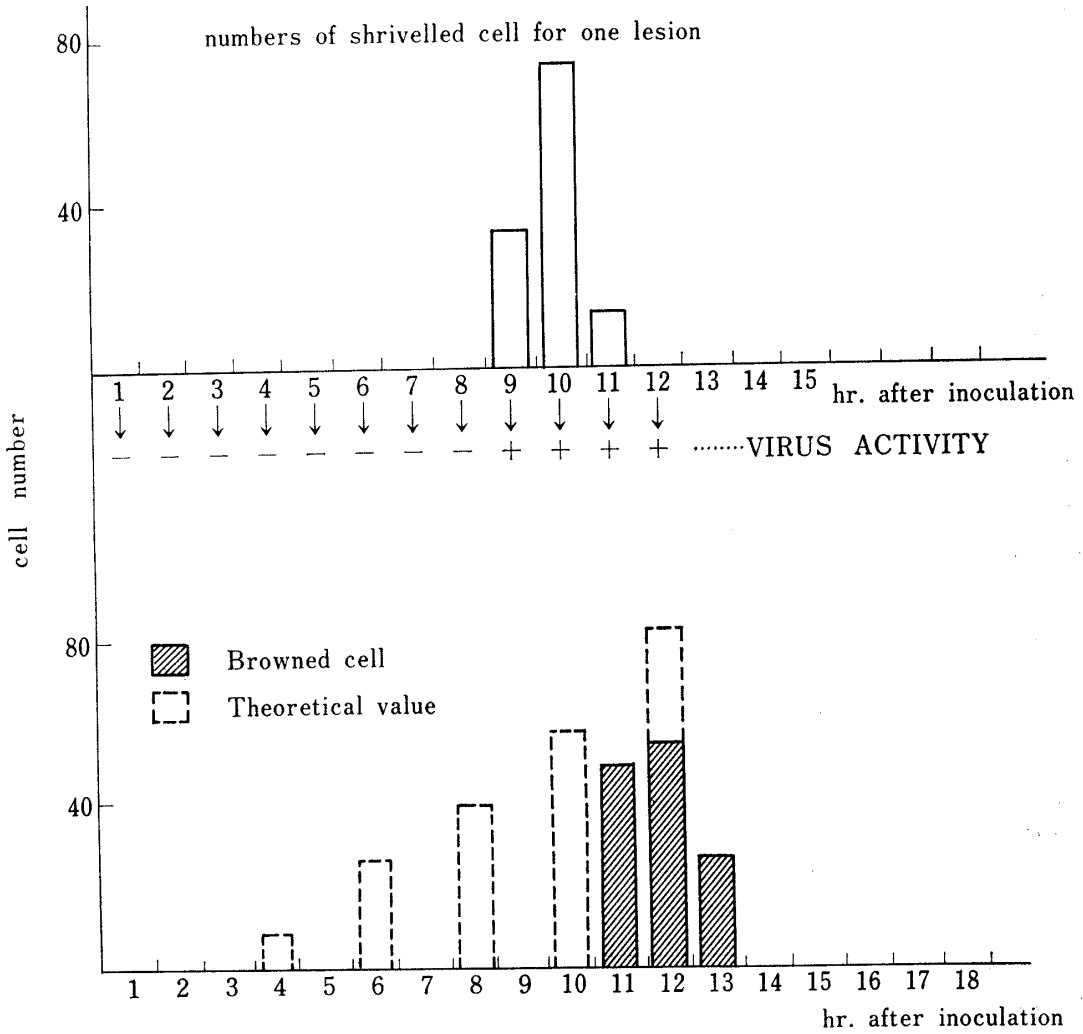


FIG. 2. Morphological change of palisade parenchyma cells in local lesion. Each values are same as Fig. 1.

TABLE 1. *Appearence of virus activity on cowpea infected by CMV.*

Periods after inoculation (hr) / Replication	5-6	9-10	13-14	17-18
i	0/1*	1/1	1/1	0/1
ii	0/1	1/1	0/1	0/1
iii	0/1	1/1	1/1	0/1

* number of infected plants number of inoculated plants tobacco was used as test plant.

cells in a lesion (Fig. 1, 2).

b) *Formation of local lesion and virus replication*

The relation between the time course of morphological and physiological changes in the infected tissue and the appearance of virus activity in this tissue were investigated. At 5–6, 9–10, 13–14 and 17–18 hours after the inoculation respectively, the inoculated cowpea leaves were harvested. Five grams of the leaves at each period were sampled and from them the virus was extracted by Scott's method (4). Virus activity of the extract was bioassayed by inoculation to tobacco as the test plant. Results are shown in Table 1. Virus activity in the infected cowpea leaf had not been recognized by 9–10 hours after the inoculation. But at 9–10 hours when the palisade cell began to shrivel, virus activity was recognized. Therefore, in the combination of CMV and cowpea, it is presumed (from this result and the above mentioned results) that the principal portion involved in virus replication and substantial portions of the necrotic local lesion formation are not epidermal cells, but mesophyll cells. Over thirteen hours after the inoculation, the extract did not occur the symptom in all test plants. At these period, the local lesion already became brown. The reason may be either inactivation of the virus or difficulty or extracting the virus from such cells and inactivation of the virus in the process of extraction by the browning of the cell.

c) *Behavior of virus in the cell during the latent period.*

As to the portion whch forms the local lesions, the shrivelled cell groups were observed in the palisade parenchyma tissue at about 8 hours after the inoculation, and then the peroxidase of the epidermal cells on this portion was activated. Before this stage, a morphological abnormality was not recognized in any portion. Moreover, a virus activity was not recognized by a bioassay. This experiment was performed to investigate the behavior of virus before the occurrence of the morphological change or the appearance of virus activity in the infected tissue by observing the inhibitive effect of infection with a treatment of high temperature and immersion in water.

1) The effect of air temperature on the formation of local lesion.

As the metabolism of a host cell is influenced extremely by high temperature treatment, the behavior of virus in the cell would also be influenced by this treatment. Therefore, it was assumed that the replication and spreading of the virus after the inoculation would be inhibited variously by degrees of air temperature. The inoculated cowpeas were kept in incubaters controlled at 20°C, 28°C, 30° and 34°C respectively, and the number of local lesions formed was estimated. The result is shown in Figure 3. The greatest number of the local lesions were formed at 28°C. The number of local lesion formed at 20° and 30°C were 60 and 70% of that at 28°C. At 34°C, the number of local lesions decreased greatly and was below 10% of that of 28°C. On the other hand extraction of the virus was attempted from the portion without the local lesion or in the vicinity of the local lesion by the method of Scott (4). But these extracts had not infectivity. Therefore, it is supposed that the virus does not replicate in any tissue except in the local lesion portion. These results indicate that decreasing of number of local lesions by 34°C temperature treatment is not caused by transforming from the local infection to the systemic infection, but by inhibition of virus replication.

2) Effect of immersion in water

One of the inoculated cowpea primary leaves was cut out and immersed in water for 24 hours. The number of local lesions formed on the leaf immersed in water (at room temperatue) were compared with the number of local lesions on the

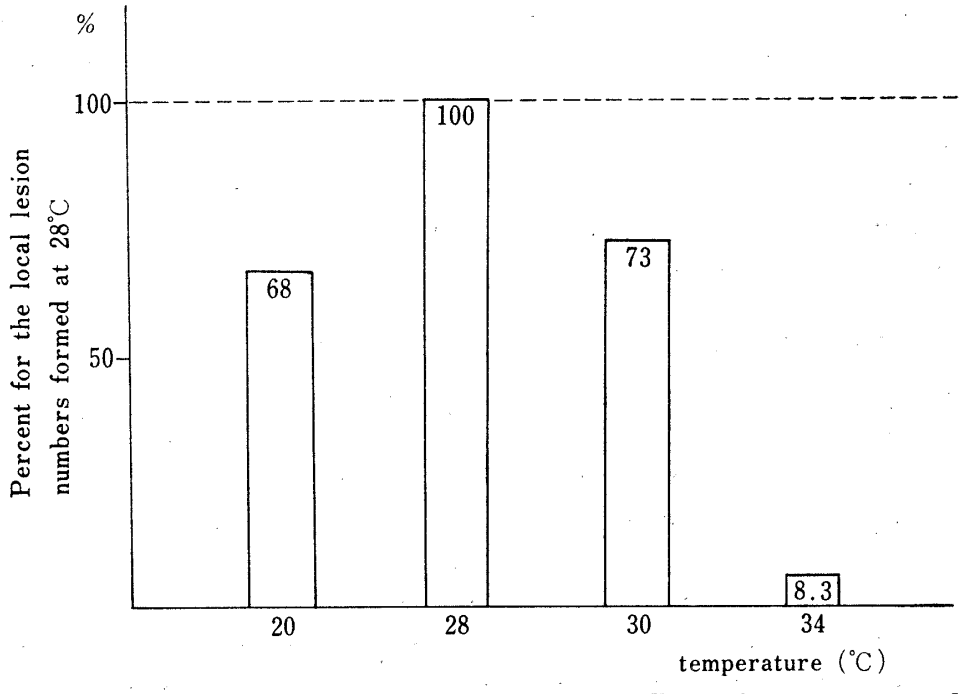


FIG. 3. Effect of temperature on the infection. 20 seedlings of cowpea were used for one treatment.

TABLE 2. *Effect of immersion in water on the infection.*

Replication / Rate of local lesions	i	ii	iii
Numbers of local lesion formed in treated leaf / Numbers of local lesion formed in untreated leaf	13/263	89/827	19/372

Five cowpeas were used, and values are an average.

untreated opposite leaf, as the control. The number of local lesions formed on the treated leaf decreased extremely in comparison with that of the untreated leaf; namely, its number was a few percent of the control as shown in Table 2. This result indicates that the infection process was inhibited remarkably by the treatment of immerson in water.

3) Relation between the infection inhibiting treatment and the time after inoculation.

In the above mentioned experiments, when the inoculated cowpea leaf was placed at 34°C or was immersed in water, the infection was remarkably inhibited. While we have reported already that the inoculated virus decoated in the primary infected cell by about 2.5 hours after the inoculation and then the infective entity spread to adjacent cells and secondary infected cells were formed (5). Therefore, in this experiment, the inoculated cowpea leaves were treated from 3 hours after the inoculation in order to avoid the effects of the early stages of infection. Treatments for inoculated leaves are as follows (Fig. 4).

i) After the inoculation, immediately cowpea leaves are transferred at 34°C and then are moved every one hour to 28°C;

ii) Leaves placed at 28°C after the inoculation, are moved to 30°C with one hour intervals;

iii) After the inoculation, leaves are immediately immersed in water and then removed from the water every hour until all leaves are removed;

iv) Leaves are immersed in water at the intervals of one hour after the inoculation;

After a given hour, the number of local lesions formed in each treatment were counted and the relation between the time after the inoculation and effect of four treatments on the infection were investigated.

a) Effect of high temperature treatment

Results are shown in Figure 5. When the inoculated leaves were placed at 34°C for less than 2 hours after the inoculation and then transferred to 28°C, the infection was not influenced. But when the inoculated leaves were placed

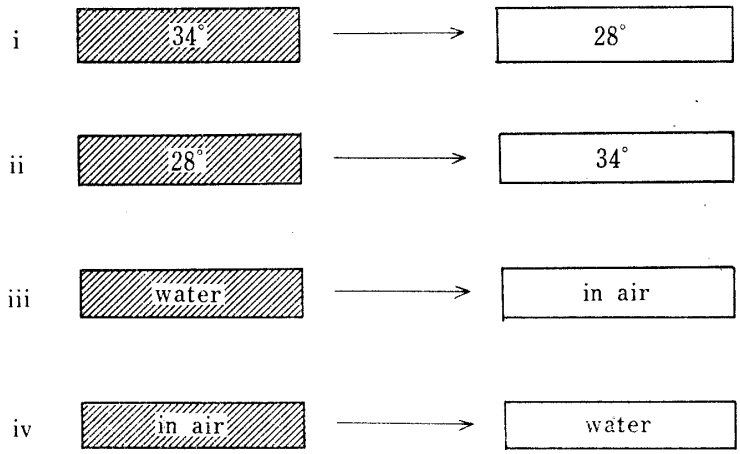
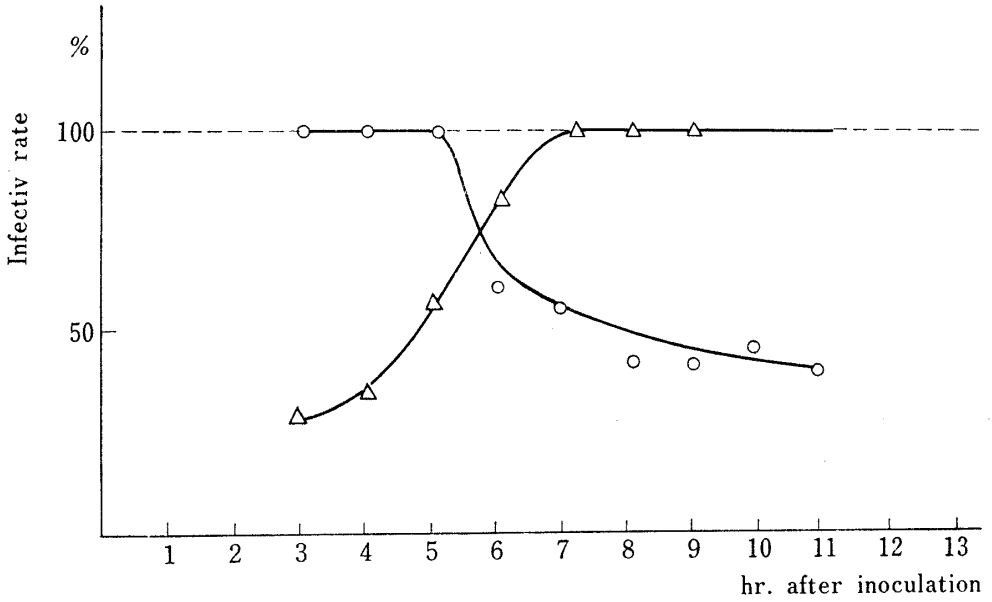


Fig. 4. The method of treatment for inoculated leaves. After the inoculation, immediately all cowpea leaves are tranferred at left condition (▩) and then a given cowpea leaves from then was moved to left condition (□) at every one hour as arrow.



○–○: Value of experiment of c), 3), i).
△–△: Value of experiment of c), 3), ii).

$$\text{Infective rate: } \frac{\text{mean number of local lesion on the treated leaf}}{\text{mean number of local lesion on the untreated leaf}} \times 100$$

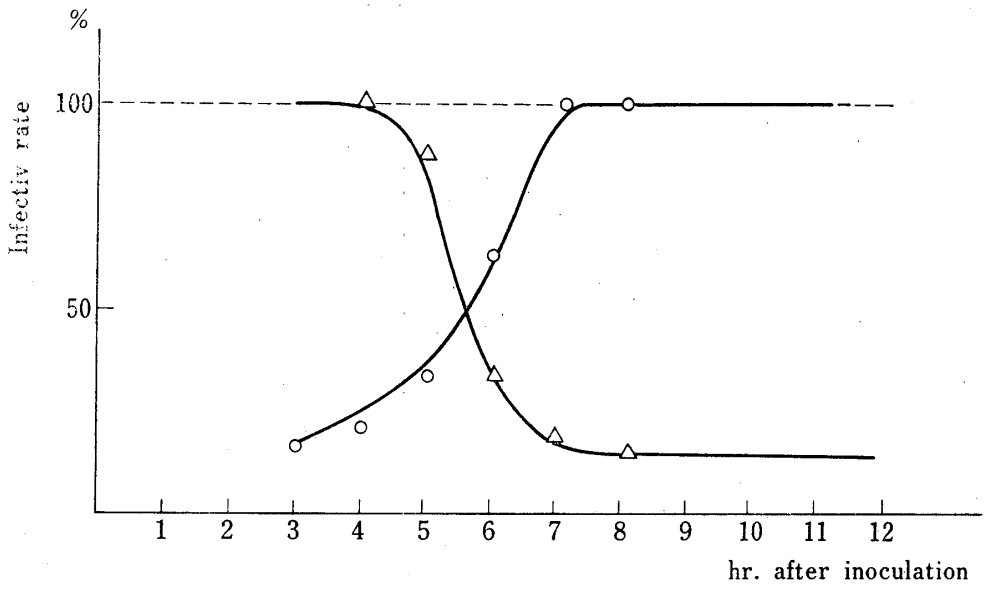
30 cowpea were used in each treatment.

Fig. 5. Effect of 34°C treatment

for 3 hours or more 6 hours at 34°C after the inoculation and then moved to 28°C, the infection was extremely inhibited. When they were placed at 34°C after 7 hours after the inoculation, the infection was not influenced.

b) Effect of immersion water

Results are shown in Figure 6. When cowpea leaves were immersed in water after 2 hours after the inoculation and then were taken out within 5 hours after the



△–△ valu of experiment of c, 3, iii.
○–○ value of experiment of c, 3, iv.

Fig. 6. Effect of the immersion in water on formation of the local lesion.

inoculation, the infection was not influenced, but when they were immersed for more than 6 hour after the inoculation, the infective rate extremely decreased. Moreover, when the inoculatd leaves were immersed in water continuously from 6 hours after the inoculation, the infective rate decreased greatly.

The above experimental results show that when the inoculated cowpea leaves were placed at 34°C or immersed in water after the inoculation, the infection was extremely inhibited according to the time of treatment. This is, on the relation between the time of treatment and the infection, when the time of treatment was within 5 hours, the infection was not influenced. On the contrary, by a treatment of more than 7 hours after the inoculation, the infection decreased greatly. Also if the leaves were placed at 28°C for 7 hours after the inoculation, the infective rate was not influenced by transferring at 34°C after that period. Therefore, the treatment of 34°C or the immersion in water would influence the behavior of the virus in the mesophyll cell in the period of from 5 to 7 hours after the inoculation.

From the results described in the previous reports, 5–7 hours after the inoculation corresponds to the time that the infective entity spread to the tertiary infected cells and so on. Namely, this period is the phase that the infective information spreads in cells, which finally compose the local lesion. When inhibitive treatment as described above, are done at this phase, virus replication is blocked in these cells, and so it seems that the formation of local lesion does not occur.

Discussion

As soon as the tissue of the host was invaded by the parasite, the invaded

cells and its adjcent cells died and the infection is inhibited. Such a phenomenon has been called as a hypersensitive reaction. For example, this phenomenon has been investigated in the infection by obligate parasites or some perthophytes. This hypersensitive reaction is caused by the mutual interaction of the host and the parasite, and it has also been thought that the local lesion formation by the infection of the virus is a kind of hypersensitive reaction. However, it is difficult to think that the hypersensitivity caused by infection of virus and fungus are produced by the same mechanism, because virus is essentially different from fungus. Whereas in the case of virus, the trace of behavior of the virus in the infected tissue is difficult to confirm, different from the experiment on fungus diseases. Therefore, study of the problem of hypersensitive reaction in virus infection has not progressed very much.

From experiments described in the third report (5), it was supposed that the infective entiry had need of about 2.5 hours (at the point of 100% on infective rate) for spreading from the primary infected cell to the secondary infected cell by the results of treatments to the inoculated epidermis. On the other hand, it was presumed that the virus spreaded from the epidermis to the mesophyll tissue at 7 hours after the inoculation from the results of experiment by epidermis stripping method. Moreover, this result shows that the infective entity reached more than two layers of the mesophyll cell, because one layer of mesophyll cell adjacent to the epidermis seemed to die, because of damage, as soon as the epidermis was stripped out. In this case, the primary infected cells are the mesophyll cells which is adjacent to the epidermis. Therefore, results obtained from the stripping method of epidermis show the time when the infective entity reached to the tertiary infected cells from the primary infected cells. Therefore, it is thought that about 7 hours are necessary to establish the tertiary infected cell after the infection of the epidermis, and the time of spreading from the secondary infected cell to the tertiary infected cell is about 4.5 hours (7 hours−2.5 hours×4.5 hours). As mentioned above, the formation of the primary infected cell, the secondary infected cell and the tertiary infected cell are different with regard to time. However, we have to consider the factors obtained from the results of this experiment which are mentioned hereafter. It was indicated already that the cell numbers consistuting the necrotic lesion formed in cowpea leaf by the infection of CMV were about 30 epidermal cells and about 120 in palisade cells. These necrotic cells are not formed successively and gradually after the inoculation. At 8–9 hours after the inoculaton, shrivelled cells in the infected palisade tissue, namely cells in the early stage of necrosis, are observed to occur suddenly. At this stage, the number of shrivelled cells in the palisade tissue corresponds to 90% of the final total number of cells of the necrotic local lesion. Also at this stage, the virus activity was first recognized. Namely, about one hundred palisade cells change morphologically almost simultaneously, and moreover the virus activity

appeared in these cell groups. The period in which the second and the third infected cell changed morphologically, consisted with the period in which the virus activity of their cells appeared. These result show that the virus did not spread successively to the surrounding cells with uniform velocity, but spread abruptly to many cells during a certain period after the invasion. Also, the virus replicated almost simultaneously in that invaded cells and the cells changed morphologically. Therefore, the time in which the virus activity appeard in the secondary and tertiary infected cells and that in which a morphological abnormality of cells appeared in the infected portion was almost equal to each other. From these observations an the previously described results, in the combination of CMV and cowpea the behavior of the virus in the tissue up to the local lesion formation would be presumed to be the infective entity produced in the primary infected cell spreading to the adjacent cells (these cells become the secondary infected cells) by 2.5 hours after the inoculation (6), and the third layer of cells (these cells become the tertiary infected cells) infected by 7 hours after the inoculation. From hereon, the new infective entity is produced abundantly by 4.5 (7–2.5) hours after it spreads to the secondary infected cells and spreads immediately to many surrounding cells. Thus, the tertiary infected cells are formed. Therefore, in the secondary and tertiary infected cells, the time of initiation of virus replication would be nearly the same. At 7 hour after the inoculation, the cells which become the primary necrotic portion (5) are decided, and by 2–3 hours later, virus activity appears in these cells; such cells change morphologically. When cowpea was inoculated with CMV and was placed under 34°C or immersed in water, the number of local lesions formed decreased greatly. With regard to the relation between the treated time after the inoculation and number of local lesions formed. When the treatment was started by 5 hours after the inoculaton, the number of local lesions decreased greatly. When the period of treatment was stopped within 5 hours after the inoculation, or treatments were done from after 7 hours after the inoculation, the number of the local lesions was not influenced. But when the treatments were started within 7 hours after the inoculation, or were continued over 5 hours after the inoculation, the number of the local lesion formed decreased. Therefore, the cause which the infection is inhibited by 34°C treatment of immersion in water suggests that the behavior of the virus at between from 5 hours to 7 hours aftar the inoculation was inhibited. This phase corresponds to the phase in which the infective entity is produced in the secondary infected cell and then tertiary infected cell is formed. It is supposed that when the cell physiology became abnormal under treatment at 34°C or immerson in the water in this phase, the secondary and tertiary infected cells which would be the subject of primary necrosis were not formed. Therefore, the formation of the local lesion was inhibited. Hirai et al investigated the inhibition of virus (TMV) infection due to Blasticidin S, and they obtained results that the infection was inhibited by the treatment

within 6 hours after the inoculation but that it was not inhibited by treatment after 8 hours (8). This relationship between the time of the treatment and the inhibition of the infection is very similar to our results.

On the other hand, a presumption that the spreading of the virus from cell to cell is performed only by replication of the whole virus in each cell, is difficult to accept from the above mentioned results. Because the virus particle is able to be found in plasmodesmata on the electron microscopic observation (9, 10, 11), the role of the virus particle for spreading can not be excluded, as cited above. It is presumed that the spreading of the infection from cell to cell is not only depend on the whole virus, but mainly any infective entity defferent from the whole virus.

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